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Mutation of the Hof1 pest domain affects cytokinesis in budding yeast

Katherine E. Stockstill

MUTATION OF THE HOF1 PEST DOMAIN AFFECTS CYTOKINESIS IN BUDDING YEAST

by

KATHERINE ELIZABETH STOCKSTILL

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Katie Shannon, Advisor
Robert Aronstam
Nuran Ercal

ABSTRACT

Cytokinesis is the division of a cell into two daughter cells. Cytokinesis failure results in polyploidy, which may contribute to tumorigenesis or cause cell death.

The *Saccharomyces cerevisiae* protein Hof1, which is required for efficient cytokinesis, is phosphorylated during mitosis. The hypothesis that MEN (mitotic exit network) dependent phosphorylation regulates Hof1 degradation, triggering completion of cytokinesis was tested. Hof1 contains a PEST sequence, which is rich in proline, glutamic acid, serine, and threonine, and is believed to be involved in protein degradation. To test the hypothesis, we created two non-phosphorylatable mutants of the Hof1 PEST domain tagged with GFP. The first mutant is a 5A which has the first five serines replaced with alanine. The second mutant is a 10A which has nine serines and one threonine replaced with alanine. Using time-lapse microscopy we have shown that phosphorylation of the Hof1 PEST domain is required to remove Hof1 from the bud neck after cytokinesis. We used Myo1-GFP to determine if lack of PEST phosphorylation affects contraction, and our results show that it does. No cytokinesis defects were seen in cells lacking PEST domain phosphorylation. Our results suggest that neither removal of Hof1 from the bud neck nor degradation of Hof1 is required for completion of cytokinesis. Our data shows that the interaction of Hof1 with Grr1, a protein that is involved with degradation, is not regulated by PEST domain phosphorylation.

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1. INTRODUCTION

1.1. CYTOKINESIS

Cytokinesis, the final step of cell division, occurs when one cell successfully splits into two cells. In animal cells and fungi this step is possible because of contraction of the actin/myosin ring (Satterwhite, Lohka et al. 1992; Fishkind and Wang 1995). During contraction, a cleavage furrow starts to ingress and reach the central spindle, at this point the cells will undergo abscission and the cytoplasm will spilt into two (Dobbelaere and Barral 2004). During cytokinesis there is an actomyosin ring as well as a septin ring present at the site of contraction (Bi, Maddox et al. 1998; Peterson and Petty 2010). Cytokinesis is both spatially and temporally controlled to prevent aneuploidy, which could result in cell death or mutations. (Vallen, Caviston et al. 2000) Cytokinesis must occur between the chromosomes and after mitosis, to ensure that the correct number of chromosomes is in each new cell.

Previous research suggests that animal cells undergo cytokinesis by contraction of the actomyosin system (Satterwhite, Lohka et al. 1992; Fishkind and Wang 1995; Rappaport 1996). Yeast, including *Schizasaccharomyces pombe* and *Saccharomyces cerevisiae*, undergo contraction based on a similar system (Marks 1985; McCollum, Balasubramanian et al. 1995; Bezanilla, Forsburg et al. 1997; Kitayama, Sugimoto et al. 1997; Bi, Maddox et al. 1998; Lippincott and Li 1998; Tolliday, Bouquin et al. 2001). However, there are at least three differences between the yeast and animal systems. The first difference involves the timing and site selection. In *S. cerevisiae* the site of cell

division is determined at the beginning of the cell cycle (Pringle, Bi et al. 1995; Drubin
and Nelson 1996). In animal cells F-actin and myosin II are centralized at the cleavage
site, which is thought to be determined by the location of the spindle, around anaphase
(Satterwhite, Lohka et al. 1992; Rappaport 1996). The second difference is that *S.
cerevisiae* have a cell wall and animal cells do not. Because of this cytokinesis in *S.
cerevisiae* is coordinated with septum formation (Bi, Maddox et al. 1998). The third
difference is that the actomyosin contractile ring is essential in animal cells but not in *S.
cerevisiae* (Rodriguez 1990; Bi, Maddox et al. 1998; Lippincott and Li 1998).

1.2. CYTOKINESIS AND CANCER

Defects in cytokinesis can lead to aneuploidy, or an abnormal number of
chromosomes, which could cause cell death or mutations (Ganem, Storchova et al.
2007). Tetraploidy, one form of polyploidy, occurs when four sets of chromosomes are
present. Tetraploidy has been proposed as a possible route to aneuploid cancer cells
(Ganem, Storchova et al. 2007). This can occur because tetraploid cells could have multi-
polar spindles, which can lead to random chromosome separation, thus forming
aneuploid cells.   The formation of aneuploid cells is important because aneuploidy is a
hallmark of cancer cells. Research has shown that tetraploid cells can form as a result of
mitotic slippage, cytokinesis failure, or viral-induced cell fusion (Ganem, Storchova et al.
2007). Cytokinesis failure resulting in tetraploid cells can occur for a couple of different
reasons. One reason cytokinesis failure could occur is because of genetic mutations. In
one study it was shown that adenomatous polyposis coli (APC) mutations, which are
found in colorectal cancers, cause cytokinesis failure by blocking the start of the

cleavage furrow (Caldwell, Green et al. 2007). Another reason cytokinesis failure could occur is because of exposure to drugs or carcinogens. By exposing mouse mammary epithelial cells to dihydrocytochalasin B, cytokinesis was transiently blocked, resulting in tumorigenesis in p53$^{-/-}$ cells (Fujiwara, Bandi et al. 2005).

1.3. BUDDING YEAST AS A MODEL ORGANISM

Budding yeast, or *Saccharomyces cerevisiae*, are used to study cytokinesis for several different reasons. To start off with they are fairly easy to work with and they can be used in several areas of research including: biochemical, molecular, cellular, and they are genetically tractable organisms. Budding yeast are able to exist as either haploids or diploids, which is useful because it allows the recessive phenotype to be seen in haploid strains. Budding yeast contain a contractile ring that is made of actin and myosin II (Bi, Maddox et al. 1998; Lippincott and Li 1998), as do animal cells. There are also several cytokinesis genes, including septins and IQGAP families, that are conserved between yeast and mammals (Hales, Bi et al. 1999).

Budding yeast are also a useful model organism because there are known division pathways in budding yeast. Genetic experiments have found two pathways. The first pathway, which is similar to cytokinesis in animal cells, uses type II myosin (Myo1), IQG1 and the formins Bni1 and Bnr1 to help assemble F actin in the ring (Bi, Maddox et al. 1998; Lippincott and Li 1998; Tolliday, VerPlank et al. 2002). The second pathway uses several different proteins including Bnr1 and Hof1 to help control cell separation and septum formation (Vallen, Caviston et al. 2000; Blondel, Bach et al.

2005). Normally the cytokinesis and septation pathways are tightly coupled (Bi 2001). In some strain backgrounds, cells can divide in the absence of Myo1 through formation of abnormal septa.

1.4. MITOTIC EXIT NETWORK

The mitotic exit network (MEN) is a signaling pathway in budding yeast that helps temporally regulate cytokinesis. Mutations in the MEN cause the cell to arrest in late mitosis with elongated spindles, high levels of mitotic cyclin, segregated chromosomes and failure to initiate cytokinesis (McCollum and Gould 2001). Not only is the MEN involved in mitotic exit and cytokinesis, but it is also required for a cell cycle check point. During the anaphase-to-telophase transition MEN regulates cell cycle progression in response to spindle alignment and elongation as well as the transfer of the nucleus from the mother to daughter cell (Luis J. Garacia-Rodriguez 2009). The MEN is made up of several components including: protein kinases (Cdc5p, Cdc15p, Dbf2p, and Dbf20p), a GTPase (Tem1p), an exchange factor (Lte1p), a protein phosphatase (Cdc14p), and a Dbf2/Dbf20-binding protein (Mob1p) (McCollum and Gould 2001). These components, especially Tem1p, Cdc15p, Dbf2p, and Dbf20p, are required for cells to maintain their viability in the absence of contractile ring function (Corbett, Xiong et al. 2006).

There have been several components of MEN that have been shown to localize to both spindle pole bodies (SPB) and the bud neck, while others only localize to one or the other. On some occasions the localization of the components determines its

function. When looking at Cdc15, research has shown that it localizes to the SPBs as well

as to the mother bud neck (Xu, Huang et al. 2000). Localization of Cdc15 to the SPBs is

not required for mitotic exit but it is required for cytokinesis (Menssen, Neutzner et al.

2001). The localization of Dbf2 and Dbf20 has also been well studied. Dbf2 and Dbf20

start off localized to the SPB and then in late mitosis they move to the bud neck,

allowing cytokinesis to occur (Frenz, Lee et al. 2000). Frenz has also shown that

localization of Dbf2 to the bud neck is dependent on septins as well as other mitotic exit

network proteins including Mob1, Cdc5, Cdc14, and Cdc15 (Frenz, Lee et al. 2000). The

targets of the MEN that regulate cytokinesis are unknown.

1.5. SEPTINS

Septins are a family of conserved GTP-binding proteins (Tolliday, Bouquin et al.

2001; Dobbelaere and Barral 2004) that have a conserved function in cytokinesis. In

budding yeast the septins form a filamentous ring beneath the cell membrane at the

bud neck site and remain there as the bud grows (Longtine, DeMarini et al. 1996). The

septins form a double ring around the bud neck that is shaped like an hourglass; this

double ring will then split into two rings at the time of cytokinesis (Haarer and Pringle

1987; Lippincott, Shannon et al. 2001; Tolliday, Bouquin et al. 2001).

Septins have several functions and are essential to cytokinesis in several

different ways. Septins are used to recruit other cytokinesis proteins to the mother-bud

neck starting in late G1 (Gladfelter, Pringle et al. 2001). They act as diffusion barriers by

compartmentalizing cytokinesis factors (Dobbelaere and Barral 2004). They are used to

delineate the boundaries of the cleavage area (Dobbelaere and Barral 2004). They are

needed for either the abscission or the digestion of the primary septum (Dobbelaere

and Barral 2004). And they are needed to help maintain the cortical factors at the site

of cleavage (Dobbelaere and Barral 2004), which helps to maintain cell polarity (Tolliday,

Bouquin et al. 2001).

1.6. THE ACTOMYOSIN RING AND THE ANAPHASE-PROMOTING COMPLEX

The actomyosin ring which is located at the bud neck is a contractile ring that is

made up of actin and myosin II and it generates the force that is required for cytokinesis

(Lippincott and Li 1998). The timing and positioning of the actomyosin ring is important

for the transmission of both genetic and organelle materials from the mother to

daughter (Lippincott and Li 1998). This assembly is controlled at various points

throughout the cell cycle (Lippincott and Li 1998), more specifically the assembly occurs

in two temporally distinct stages (Tolliday, Bouquin et al. 2001). The first step occurs

early in the cell cycle at the G1/S transition, when Myo1 assembles at the bud site

shortly after the appearance of the septin ring (Tolliday, Bouquin et al. 2001). The

second step, which occurs during late anaphase, involves the recruitment of actin

filaments to the Myo1 ring (Tolliday, Bouquin et al. 2001). The recruitment of actin

filaments is dependent upon the IQGAP family member Cyk1/IQG1 and the formins Bnr1

and Bni1 (Lippincott and Li 1998; Tolliday, Bouquin et al. 2001; Tolliday, VerPlank et al.

2002). In yeast cells, as well as in mammal cells, the actomyosin ring disassembles and

its components are removed from the ring during contraction and then disassembled

completely after (Tully, Nishihama et al. 2009). Previous research has also shown that

the anaphase-promoting complex (APC) is important for the removal of proteins from the actomyosin ring (Tully, Nishihama et al. 2009). The APC is a ubiquitin ligase that controls progression through mitosis by targeting specific proteins for degradation (Tully, Nishihama et al. 2009).

In order to study the APC and actomyosin ring relationship, a yeast strain was made in which Cdh1, (the APC activator) was deleted. In these cells the actomyosin ring contracted at a normal rate, but the ring components were not disassembled normally during or after contraction (Tully, Nishihama et al. 2009). This showed that the APC is required for actomyosin ring disassembly and it suggested that the APC may help promote successful abscission by completely removing the actomyosin ring components from the division site (Tully, Nishihama et al. 2009).

1.7. MYO1

Type II myosin heavy chain is responsible for producing the contractile force that is found in muscle cells. In budding yeast the localization of Myo1, which is the only type II myosin in budding yeast, requires septin function (Bi, Maddox et al. 1998). The type II myosin heavy chain is controlled by two light chains, the essential light chain (ELC) and the regulatory light chain (RLC) (Luo, Vallen et al. 2004). Mlc1, the essential light chain, interacts with Myo1 to coordinate actin ring formation, while Mlc2, the regulatory light chain, interacts with Myo1 to help with ring disassembly (Luo, Vallen et al. 2004).

Myo1, which is part of the actomyosin ring, has been shown to play an important part in cytokinesis. Myo1 starts off localized to the bud neck early in the cell cycle

(Watts, Miller et al. 1985) as either a single or double ring, at which point it overlaps with the septin ring (Lippincott and Li 1998). During anaphase actin is recruited, and the completed actomyosin ring is present until cytokinesis when it contracts to a single dot and then disappears (Lippincott and Li 1998; Tully, Nishihama et al. 2009). The Myo1 ring has also been shown to be present in cells with buds of all sizes (Lippincott and Li 1998). There has been quite a significant amount of research done looking at Myo1. The research has shown that when Myo1 is completely knocked out of a strain, the cells give rise to chain phenotype, showing that cytokinesis is not occurring as successfully (Bi, Maddox et al. 1998; Lippincott and Li 1998). Research has also shown the strains lacking Myo1 fail to form the actin ring (Bi, Maddox et al. 1998).

1.8. HOF1

Hof1 is a SH3 domain containing protein that localizes to the bud neck and is required for efficient cytokinesis. The localization of Hof1 to the bud neck requires the presence of the septins (Bi, Maddox et al. 1998; Lippincott and Li 1998; Vallen, Caviston et al. 2000). Hof1, also known as Cyk2, is a member of the PCH family of proteins. The Pombe Cdc15 homology (PCH) proteins make up a family, in which its members are found in diverse eukaryotic organisms, including yeast and mammals (Lippincott and Li 2000). Early in mitosis Hof1 forms a single ring at the mother bud neck followed by another Hof1 ring at the daughter bud neck (Vallen, Caviston et al. 2000). Later in mitosis Hof1 is phosphorylated and the double rings form a single ring which contracts, after septum formation this contracted single ring splits into two rings and disappears with cell separation (Vallen, Caviston et al. 2000).

Research on Hof1 has suggested that it plays a role in both cell division pathways: actomyosin ring dynamics and septum formation. The scientific community has different opinions on the role of Hof1. Many people think that Hof1 is involved in both actomyosin ring dynamics and septum formation; others think that it is only involved in one or the other, and yet others are still undecided.   Lippincott and Li were able to use immunolocalization and time lapse video microscopy to look at Hof1 at the bud neck. They showed that the Hof1 double ring merges into a single ring after the completion of the spindle elongation and that Hof1 shifts from the septin ring to the actomyosin ring during anaphase (Lippincott and Li 1998). They also created a null allele of Hof1 in which the cells had a cytokinesis defect (Lippincott and Li 1998). In these same cells they looked at the actin and myosin rings, which were able to form properly (Lippincott and Li 1998). Lippincott and Li showed that cytokinesis failure occurred in cells where Hof1 was deleted by looking at Myo1 contraction (Lippincott and Li 1998). In this study they compared Myo1 contraction in Hof1 wild type cells to the Myo1 contraction in ΔHOF1 cells. By using time-lapse microscopy and comparing fluorescence intensity they showed that in wild type cells the Myo1 ring increased as the ring contracted and then decreased until contraction was over, however in ΔHOF1 cells Myo1-GFP decreased as soon as contraction started and sometimes contracted asymmetrically (Lippincott and Li 1998). When looking at the rate of contraction, the wild type cells contracted at a rate of 0.26 μm/min where as the ΔHOF1 cells contracted at 0.10 μm/min (Lippincott and Li 1998). By using Nomarski imaging they were able to see vesicle movement through the bud neck in ΔHOF1 but not in wild type cells,

showing that cytokinesis had failed (Lippincott and Li 1998). Based off of their results Lippincott and Li not only showed that cytokinesis failure did occur but also that Hof1 is needed to help with Myo1 stability during contraction (Lippincott and Li 1998). When taken together this data shows that Hof1 interacts with the actomyosin ring, but is not required for its formation (Lippincott and Li 1998).

Vallen and Bi suggest that in the absence of an actomyosin ring, cell division can still occur because of septum formation, and that Hof1 is involved in septum formation for three reasons (Vallen, Caviston et al. 2000). The first reason is that chitin, the cell wall component, in HOF1 null cells is delocalized at nonpermissive temperatures (Kamei, Tanaka et al. 1998; Lippincott and Li 1998; Vallen, Caviston et al. 2000). The second reason is that only proteins that are components of chitin synthase III or involved in recruiting CSIII to the bud neck localize to the mother-side of the bud neck, and Hof1 is one of these proteins (Chuang and Schekman 1996; DeMarini, Adams et al. 1997). And the third reason is that only a defect in cell separation was caused by the deletion of HOF1 (Vallen, Caviston et al. 2000). To further support the idea that Hof1 is involved in septum formation Vallen and Bi looked genetically at the Hof1-Myo1 interaction. In their experiment they crossed strains in which MYO1 was deleted from one and HOF1 was deleted from the other, resulting in 18 out of 19 of the double mutants not growing at 23°C, showing that the double deletions are synthetically lethal (Vallen, Caviston et al. 2000). By showing that MYO1 and HOF1 deletions are synthetically lethal, they concluded that they must likely define different pathways in cytokinesis (Vallen, Caviston et al. 2000).

By using time-lapse microscopy and western blots on a Hof1-HA strain Vallen and Bi were able to show that Hof1 bud neck localization is cell cycle dependent (Vallen, Caviston et al. 2000), at 60 minutes, which is just before or during anaphase, is where the levels of Hof1 peaked. They also showed by western blot that the change in the Hof1 mobility was due to hyperphosphorylation, which occurs after late anaphase/telophase, and most likely during cytokinesis or cell separation (Vallen, Caviston et al. 2000). This data suggests that for Hof1 to become a single ring or be removed from the bud neck, it has to be hyperphosphorylated (Vallen, Caviston et al. 2000).

As well as having different roles in cytokinesis, Hof1 also interacts with several different proteins including Inn1, Bnr1, and Verprolin. Inn1 is a protein that is needed for primary septum formation, and research has shown that it interacts with Hof1 to promote primary septum formation in coordination with the actomyosin ring contraction (Nishihama, Schreiter et al. 2009). Bnr1, a member of the formin family, is a protein that is needed to help promote the assembly of actin filaments. Bnr1, which also localizes to the bud neck (Kamei, Tanaka et al. 1998), has been shown to directly interact with Hof1 to regulate cytoskeletal functions (Kamei, Tanaka et al. 1998; Vallen, Caviston et al. 2000). Verprolin (Vrp1) is a proline rich protein that associates with actin (Ren, Wang et al. 2005). Loss of Vrp1 results in loss of actin patch polarization and cytokinesis defects in which the cells arrest with a single large bud and no bud neck septum (Ren, Wang et al. 2005). Research has shown that Vrp1 interacts with Hof1, at 37°C, by binding to the Hof1 SH3 domain (Ren, Wang et al. 2005).

1.9. THE HOF1 PEST DOMAIN

Research by Blondel *et al* has shown that Hof1 protein levels decrease at the end of mitosis, and that this down regulation is needed for efficient contraction of the actomyosin ring and cell separation during cytokinesis (Blondel, Bach et al. 2005). By using western blots these authors suggest that Hof1 is degraded at the end of mitosis and that the degradation is dependent on it's PEST Domain and Grr1 (Blondel, Bach et al. 2005). Grr1 is a Skp1-Cullin-F-box protein complex (SCF) that is found in the nucleus and the cytoplasm and it accumulates at the bud neck late in mitosis (Blondel, Galan et al. 2000). The SCF protein complex is an E3 ligase that helps control the cell cycle by ubiquitin-dependent degradation of cell cycle regulators including proteins (Blondel, Bach et al. 2005). A genome wide two-hybrid screen was done by Ito et al, showing that there is a interaction between Grr1 and Hof1 (Ito, Chiba et al. 2001). Blondel *et al* also confirmed the in vivo Hof1–Grr1 interaction by use of the bimolecular fluorescence complementation system (Blondel, Bach et al. 2005). It has been shown that the activation of MEN is necessary for the recruitment of Grr1 to the bud neck which suggests that Grr1 may degrade a substrate that is involved in cytokinesis (Blondel, Bach et al. 2005). However, Hof1 was able to localize to the bud neck in Grr1Δ cells normally (Blondel, Bach et al. 2005).

The PEST Domain is a short sequence of polypeptides that is rich in proline, glutamic acid, serine and threonine, and it is involved in protein degradation (Rogers, Wells et al. 1986). A PEST-Find program has been used and has located a PEST domain between amino acids 418-438 of Hof1 (Blondel, Bach et al. 2005). Hof1 is a known

phosphoprotein, and has been shown to be phosphorylated at the first serine of the

PEST domain (Bodenmiller, Campbell et al. 2008). In order to study the Hof1-Grr1

interaction Blondel *et al* made a Hof1 mutant that lacked the PEST domain. By making

and using this mutant they were able to show a significant amount of data on the

interaction. Using time-lapse microscopy they were able to show that Hof1 is degraded

during the G1 phase, but hof1ΔPEST is not (Blondel, Bach et al. 2005). By western blots

they were able to look at the phosphorylation of Hof1 and hof1ΔPEST, which suggested

that the PEST domain of Hof1 is important for phosphorylation (Blondel, Bach et al.

2005). By using time-lapse microscopy to look at Myo1-GFP they noticed a difference

between the Hof1 wild type and the hof1ΔPEST cells. Their hof1ΔPEST cells were

delayed in the contraction of the actomyosin ring (Blondel, Bach et al. 2005). Overall

Blondel's research suggests that Hof1 is targeted for degradation by Grr1 and that this

degradation is dependent on the PEST domain and it regulates myosin contraction.

This data led to the hypothesis that MEN dependent phosphorylation of the Hof1

PEST domain regulates Hof1 degradation and Myo1 contraction.

2. RATIONALE AND DESIGN

2.1. INTRODUCTION

Actomyosin ring contraction and septum formation lead to cell division. In eukaryotic organisms the role of the actomyosin ring in cytokinesis is highly conserved (Lippincott, Shannon et al. 2001). In *Saccharomyces cerevisiae*, budding yeast, the mitotic exit network regulates mitotic exit and cytokinesis (Garcia-Rodriguez, Crider et al. 2009). It has been shown that MEN activity is required for the phosphorylation of Hof1 during mitosis (Vallen, Caviston et al. 2000; Blondel, Bach et al. 2005). Hof1 has been shown to be phosphoralyated at the first serine of the PEST domain (Bodenmiller, Campbell et al. 2008). Blondel *et al* has shown that Hof1 contains a PEST domain which is involved in the degradation of Hof1 upon mitotic exit (Blondel, Bach et al. 2005). Deletion of the PEST domain slowed Myo1 contraction (Blondel, Bach et al. 2005). This led to the hypothesis that MEN regulates myosin contraction through phosphorlyation of Hof1. To test this hypothesis two PEST domain non-phosphorylatable mutations were made and analyzed by looking at cell morphology, Hof1-Grr1 interaction, Hof1-GFP dynamics, and Myo1-GFP dynamics.

2.2. METHODS

2.2.1. Strains and Media. All *S. cerevisiae* strains were derived from either S288C or W303 backgrounds. All of the yeast strains used in this study can be found in Table 1.1. Yeast cells were either grown in YPD (yeast extract peptone dextrose), YPGR

(Yeast extract peptone ribose and galactose) or on – HIS, – LEU, – TRP or – URA drop

out agarose plates (Guthrie 1991).

Table 1.1. Yeast Strains used in this study

Strain	Genotype	Background	Source	Reference
KSY 35/ RLY292	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3	S288C	R Li	(Lippincott and Li 1998)
KSY 108	MAT a/α his3/his3 leu2/leu2 ura3/ura3 lys2/lys2 Hof1Δ: HIS3/HOF1 YGR250CΔ:URA3/YGR250C pLP2(Hof1-GFP, LEU2)	S288C	This work	
KSY 158	MAT a Ura3 HOF1Δ:HIS3 hof1-GFP, LEU2 (pLP2)	S288C	This work	
KSY 166/ RLY416	MAT a/α his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade2/ade2 can1/can1 Hof1Δ:HIS3/HOF1	W303	R Li	(Lippincott and Li 1998)
KSY 168	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3 Hof110A-GFP, LEU2 (pJP3)	S288C	This work	
KSY 172	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3 pJP1 (HOF1-GFP(mutP1), LEU2) Hof5A-GFP	S288C	This work	
KSY 188/ YEF2016	MAT a his3 leu2 lys2 trp1 ura3 HOF1:HA::HIS3		Bi Lab	(Vallen, Caviston et al. 2000)
KSY 190/ YEF1986	MAT a/α his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 HOF1:GFP::Kan/HOF1:GFP::Kan		Bi Lab	(Vallen, Caviston et al. 2000)
KSY 240	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3 pLP1 (HOF1-myc, LEU2)	S288C	This work	
KSY 241	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3 pRW1 (HOF1-P1-myc, LEU2)	S288C	This work	
KSY 242	MAT α Δhof1:HIS3 pRW1 (HOF1-P1-myc, LEU2)	W303	This work	
KSY 258	MAT a his 3 leu2 ura3 lys2 Δhof1:HIS3 pLP1 (HOF1-myc, LEU2) pBM200 (GAL-GRR1-GFP, URA 3)	S288C	This work	
KSY 259	MAT a his 3 leu2 ura3 lys2 Δhof1:HIS3 pRW1 (HOF1-P1-myc, LEU2) pBM200 (GAL-GRR1-GFP, URA3)	S288C	This work	

Table 1.1. (Continued)

KSY 260	MAT a his3 leu2 lys2 trp1 ura3 HOF1:HA::HIS3 pBM200 (GAL-GRR1-GFP, URA3)		This work	
KSY 267	MAT αhis3 leu2 lys2 trp1 ura3 HOF1:GFP::Kan		This work	
KSY 268	MAT α Δhof1:HIS3 pRW1 (HOF1-P1-myc, LEU2) pKT36 (MYO1-GFP:TRP1)	W303	This work	
KSY 276	MAT a/α his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade2/ade2 can1/can1 Hof1Δ:HIS3/HOF1 pLP1 (HOF1-myc, LEU2)	W303	This work	
KSY 297	MAT a his3 leu2 ura3 trp1 ade2 can1 Hof1Δ: pLP1 (HOF1-myc, LEU2)	W303	This work	
KSY 319	MAT a his3 leu2 ura3 trp1 ade2 can1 Hof1Δ: pLP1 (HOF1-myc, LEU2) pkt36 (MYO1-GFP:TRP1)	W303	This work	
KSY 328	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3 pKES2 (HOF1-P3-myc, LEU2)	S288C	This work	
KSY 330	MAT a his3 leu2 ura3 lys2 Δhof1: HIS3 pKES2 (HOF1-P3-myc, LEU2) pBM200 (GAL-GRR1-GFP, URA3)	S288C	This work	
KSY 331	MAT a Δhof1:HIS3 pKES2(HOF1-P3-myc, LEU2)	W303	This work	
KSY 335	MAT a Δhof1:HIS3 pKES2(HOF1-P3-myc, LEU2) pkt36 (MYO1-GFP:TRP1)	W303	This work	

2.2.2. Analysis of Cell Morphology. Cells were cultured overnight in 5 ml

YPD at room temperature (22-25°C). The cells were then centrifuged and resuspended

in 5ml of YPD prewarmed to 37°C and were allowed to continue growing at 37°C for 5-6

hours. As described by Lippincott and Li (Lippincott and Li 1998) 670μl of 37%

formaldehyde was added to the cultures and incubated at room temperature on a roller

for 1 hour. The cells were centrifuged and the media was removed. The cells were

washed twice with 1ml PBS. The cells were then washed with 1ml Sorbitol Buffer (1M

Sorbitol in 50mM KPO$_4$, pH 7.5). 100µl of cells were removed and centrifuged. Cells

were then placed on a slide for microscopic examination, or zymolase treated before

examination. For zymolase treatment, cells were resuspended with 1ml of Sorbitol

Buffer with 0.2mg/ml zymolyase and 2mM DTT and incubated at 37°C for 10 minutes

before being put on ice. For each strain and treatment, 200 cells were counted and

scored as chains if they contained 3 or more connected cell bodies.

2.2.3. Live Cell Imaging. Agarose pads are created as described by Waddle

et al (Waddle, Karpova et al. 1996). Briefly, pads are made by placing 170 ml of 2%

agarose dissolved in –TRP media on a slide. The agarose is allowed to dry, 5 µl of cell

culture is added, a cover slip is put on and sealed with valap (Vaseline, lanolin, paraffin).

The cells are then viewed using the Olympus Inverted Epifluorescent Microscope with a

100X Plan Apo NA 1.4 DIC Objective. FITC (EX 482/35 506DM EM 536/40) filter was used

(Brightline). Images were captured with a Hamamatsu ORCA285 CCD camera. Shutters,

filters, and camera were controlled using SlideBook software (Intelligent Imaging

Innovations, Denver, CO).

2.2.4. Fluorescence Intensity Measurements. Data analysis was

performed using the Slidebook software (Intelligent Imaging Innovations, Denver, CO)

based on the method described by Tully et al (Tully, Nishihama et al. 2009). A region

was drawn around the bud neck fluorescence; this region was then duplicated twice.

One of the duplicated regions was set in an area outside the cells as the background,

while the other was moved into the cytoplasm. The software would then measure the

average fluorescence for each region and subtract the background, throughout the time

lapse series. These values were normalized by taking the intensity of the frame before contraction and dividing.

2.2.5. LiAc Yeast Transformation. According to Gietz *et al's* . procedure yeast transformations were performed (Gietz, St Jean et al. 1992). 5ml YPD cultures were grown overnight. The cultures were spun down, and the cells were resuspended in 5ml sterile water. The cells were then spun down and resuspended in 5ml TEL (TE-Tris and EDTA, LiAC-Lithium Acetate, sterile Millipore water). The cells were then spun down and resuspended in 100µl TEL. 100 µl of the yeast suspension was put into a sterile eppendorf, into which, 10µl salmon sperm DNA and 2µl plasmid DNA was added. 350µl PLATE (PEG-polyethylene glycol, TE, LiAC) was immediately added and mixed completely, the yeast suspension was then incubated at 30°C for 30 minutes. 50µl DMSO (dimethyl sulfoxide) was then added and mixed to the yeast suspension, it was then heat shocked at 42°C for 15 minutes. The yeast suspension was then spun down, and the supernatant was poured off. The pellet was then resuspended in 1ml sterile 1X TE. The cells were then plated on appropriate drop-out media, and incubated at 30°C for two-three days.

2.2.6. Yeast Protein Extracts. The protocol used is a modified version of Rigaut's (Rigaut, Shevchenko et al. 1999). A 50ml YPD culture was grown overnight. The cells were centrifuged and washed with 10ml cold UB Buffer (100mM Hepes, 1M KCl, 1M $MgCl_2$, 100mM EGTA, dH_2O) or NP-40 Buffer (15mM Na_2HPO_4, 10mM NaH_2PO_4-H_2O, 1% NP-40, 150mM NaCl, 2mM EDTA, 50nM NaF, 0.1mM Na_3VO_4, dH_2O). The pellet was resuspended with a volume of either NP-40 buffer or UB buffer, PIs (100X), DTT, and

PMSF (100X) that was about twice the volume of the cell pellet. 500μg of glass beads were added to the cells and chilled on ice for 10 minutes. The cell suspension was vortexed 10 times (30 seconds on the vortexer and 1 minute on ice). The cell suspension was centrifuged at 4°C on high speed for 30 minutes. The sup, or extract, was removed from the beads and flash frozen in 100μl aliquots using liquid nitrogen.

2.2.7. Co-Immunoprecipitation.   2μl of antibody (either mouse monoclonal anti-HA 16B12, mouse monoclonal anti-myc 9E10 (Covance) or mouse monoclonal anti-GFP (B-2) (Santa Cruz Biotechnology)) was added to 100μl yeast extract. The extract was then incubated at 4°C for 1 hour on a rotator. 25μl of protein A beads (Santa Cruz Biotechnology) pre-equilibrated in extract buffer were added to the extract and incubated an additional hour at 4°C on a rotator. The extracts were then quickly centrifuged at 4°C. The supernatant was then removed from the beads, 20μl of sup was kept and mixed with 2X Lamelli sample buffer. The beads were resuspended in 100μl ice cold UB + 0.1% Triton Buffer or NP-40 Buffer and centrifuged, the wash was removed and the step was repeated twice. 40μl of 1X Lamelli sample buffer was then added to the beads and boiled for 5 minutes.

2.2.8. Western Blotting. Protein samples were separated on 7.5% SDS PAGE gels. Primary antibodies, mouse monoclonal anti-HA 16B12 and mouse monoclonal anti-myc 9E10 (Covance) were used at (1:1000) and mouse monoclonal anti-GFP (B-2) (Santa Cruz Biotechnology) was used at (1:500). Goat anti-mouse secondary antibody conjugated to HRP (Jackson ImmunoResearch Laboratories, Inc.) was used at (1:5000).

The blot was then developed by using an ECL kit (manufacturer). A picture is then taken

of the blot using imager and software.

2.2.9. QuikChange Site-Directed Mutagenesis. To make the Hof1 PEST

mutants, pLP1 or pLP2 was mutated using the Stratagene QuikChange II XL Site-Directed

Mutagenesis Kit per manufacture instructions. To make the 5A mutant primers P1 F&R

were used creating pRW1or pJP1. To make the 10A mutant, pRW1 or pJP1 was further

mutated with the P2 F&R primers to make pKES1 or pJP2. pKES1 or pJP2 were then

mutated with the P3 F&R primers to make pKES2 and pJP3. All plasmids and primers

used in this study can be found in tables 1.2 and 1.3 respectively.

Table 1.2. Plasmids used in this study

Plasmid	Description	Source	Reference
pLP3	HOF1-GFP, LEU2	R Li	(Lippincott and Li 1998)
pLP1	HOF1-myc, LEU2	R Li	(Lippincott and Li 1998)
pJP1	HOF1 5A-GFP, HIS3	This work	
pJP2	HOF1 mutant P2 GFP, HIS3	This work	
pJP3	HOF1 10A-GFP, HIS3	This work	
pRW1	HOF1 5A-myc, HIS3	This work	
pKES1	HOF1 mutant P2 myc, HIS3	This work	
pKES2	Hof1 10A-myc, HIS3	This work	
pKT36	Myo1-GFP, TRP1	R Li	(Lippincott, Shannon et al. 2001)
pMB200	GAL-GRR1-GFP, URA3	Blondel	(Blondel, Galan et al. 2000)

Table 1.3. Primers used in this study

Primers	Sequence
HOF1-P1-F	GGTGCATAAAAGAAATCAAGCTCTCGCCGCACCAGCAGAATCAAG
HOF1-P1-R	CTTGATTCTGCTGGTGCGGCGAGAGCTTGATTTCTTTTATGCACC
HOF1-P2-F	CACCAGCAGAAGCAGCTGCTGCTAATCCAACGGATTTTAGCC
HOF1-P2-R	GGCTAAAATCCGTTGGATTAGCAGCAGCTGCTTCTGCTGGTG
HOF1-P3-F	GCTAATCCAGCGGATTTTGCCCACATCAAAAAGAGAC
HOF1-P2-R	GTCTCTTTTTGATGTGGGCAAAATCCGCTGGATTAGC
HOF1-SEQ2	GATACACGAAGTGGGCGCAATTATCC
HOF1-SEQ4	CATTGCCATCTCCAGAAGTC

The PCR program is as follows:

95°C for 6minutes, 1 cycle
98°C for 1 minute, 18 cycles
55°C for 1 minutes, 18 cycles
65°C for 20 minutes, 18 cycles
72°C for 7 minutes, 1 cycle
4°C hold

2.2.10. Plasmid Preps. Minipreps on the Site-Directed Mutagenesis

transformation colonies were done according to the Wizard Plus SV Minipreps DNA

Purification System protocol. A Thermo Scientific nanodrop was then used to measure

the amount of DNA in each miniprep.

2.2.11. Sequencing. Sequencing was done by the MS&T cDNA Center to

confirm that the mutagenesis worked properly. For sequencing Hof1 Seq2 and Hof1

Seq4 were used.

2.2.12. TTest. Microsoft excel was used to perform a standard TTest. 2-tailed

type3 standard deviation was calculated using Microsoft excel.

2.2.13. Acknowledgements. Acknowledgements would like to be given to

the labs that have donated strains and plasmids for the use in this study, including

Blondel Lab, Bi Lab and Li Lab.

3. RESULTS

3.1. MUTANTS LACKING PEST DOMAIN PHOSPHORYLATION RESCUE THE CYTOKINESIS DEFECT OF THE HOF1Δ

It is known that Hof1 is needed for efficient cytokinesis, and other research has suggested that a Hof1ΔPEST strain is not able to undergo efficient cytokinesis (Blondel, Bach et al. 2005). In order to test the role of phosphorylation, strains were made in which the hof1 PEST domain was mutated to prevent phosphorylation. Two mutant alleles were made (hof1 5A and hof1 10A) by site directed mutagenesis in which the first 5 serines were mutated to alanine (5A) and all 9 serines and one thereoine in PEST domain (10A) were mutated to alanine (Figure 1.1.). The amino acids were changed to alanine because alanine cannot be phosphorylated.

To determine if the mutations had an effect on cytokinesis or septation cell morphology analysis was performed on hof1 wt, hof1Δ, hof1 5A, and hof1 10A strains as described in design. Since HOF1Δ has a temperature sensitive phenotype, cells were grown at 37°C, then fixed and counted with and without zymolase. The zymolase treatment is done to remove the cell wall, to ensure that the defect is a cytokinesis defect and not a cell wall defect. The analysis showed that both with and without zymolase treatment the HOF1Δ had the greatest amount of chains while hof1 wt, hof1 5A, and hof1 10A all had similar amounts of chains (Figure 1.2). A chain is defined as a cell with 3 or more buds that have not separated, showing a cytokinesis defect. A T-Test was done showing that there was a significant difference (P ≤ 0.05) between the HOF1Δ

and the other strains both with and without zymolase, but not among the hof1 wt, hof1
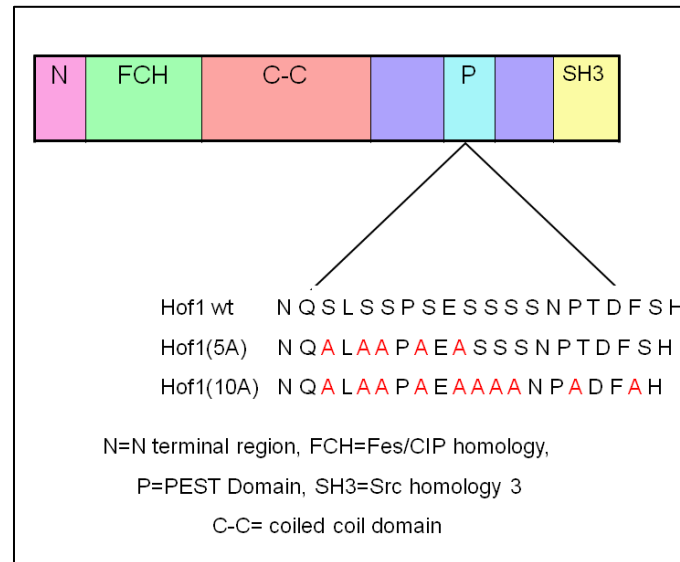
5A, or hof1 10A.

Figure 1.1. PEST Domain Mutations. The above figure represents the Hof1 PEST Domain. Site directed mutagenesis was used to mutate the wild type into the Hof1 5A and Hof1 10A strains. The first five serines were mutated to alanine in the 5A strain. In the 10A strains the first nine serines and one threonine were mutated to alanine.

To determine if hof1 5A and hof1 10A could complement the HOF1Δ, plasmids

containing wild type, 5A, and 10A were introduced into the ΔHOF1 strain and cell

growth at room temperature and 37°C were compared. The 5A and 10A cells grew as

well the wild type and completely complemented the growth defect of ΔHOF1 at 37°C
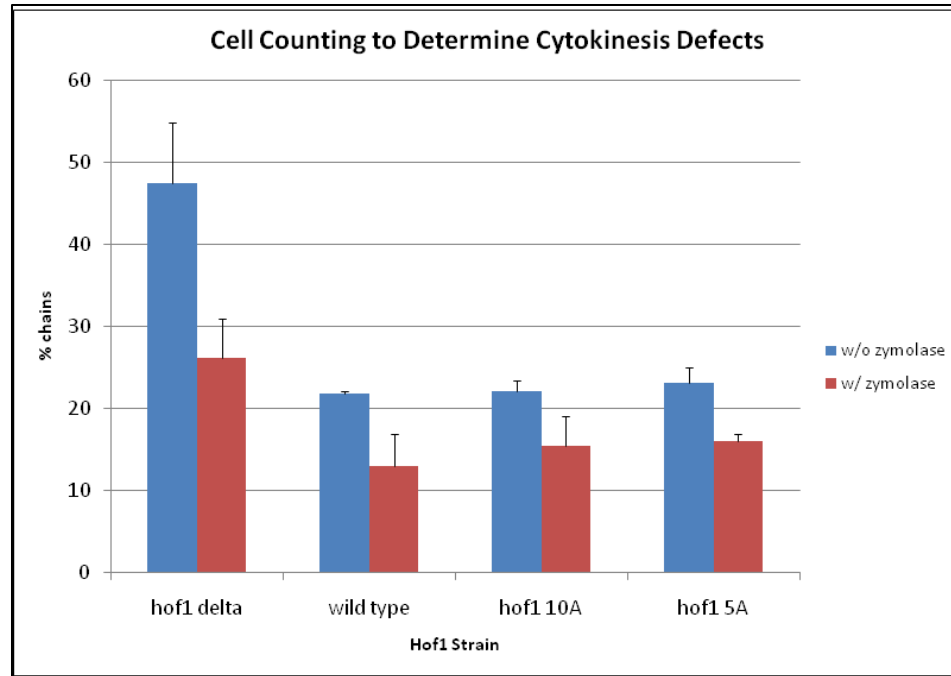
(Figure 1.3 and 1.4).

Cell Counting to Determine Cytokinesis Defects

Figure 1.2. Cell Counting of Hof1Δ, Wild Type, Hof1 10A, and Hof1 5A cells. The cells (KSY 35, KSY 158, KSY 168 and KSY 172 respectively) were grown overnight at room temperature and then at 37°C for 5-6 hours before fixation. Cells were counted with and without zymolase treatment. 200 cells from each treatment were counted. A chain is defined as 3 or more cell bodies that are together. The experiment was repeated four times and averages are shown. T-test results have shown that there is a significant difference in chains between hof1Δ and wild type, hof1 10A, and hof1 5A both with and without zymolase.
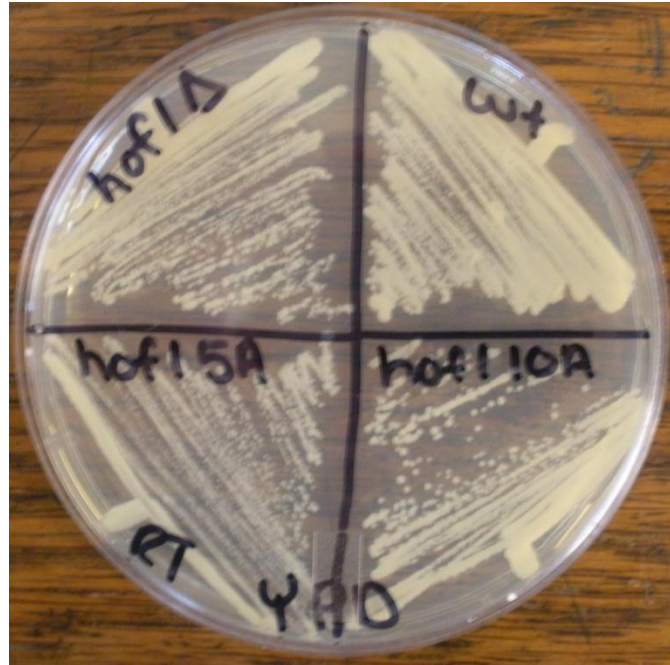
Figure 1.3. Complementation of Hof1 Temperature Sensitivity at Room Temperature. Cells were streaked to YPD plates and grown for 3 days at room temperature (≈ 25°C). Wild type, 5A, and 10A all grew at the same strength.

Figure 1.4. Complementation of Hof1 Temperature Sensitivity at 37°C. Wild type, 5A, and 10A all grew at 37°C for 3 days and were able to complement the hof1Δ growth defect.

3.2. PHOSPHORYLATION OF THE HOF1 PEST DOMAIN IS REQUIRED TO REMOVE HOF1 FROM THE BUD NECK AFTER CONTRACTION

Vallen *et al* suggested that the hyperphosphorylation of Hof1 is needed for Hof1 to leave the bud neck or to become a single ring structure (Vallen, Caviston et al. 2000). Later research suggests that the Hof1 PEST domain is important for phosphorylation (Blondel, Bach et al. 2005). Blondel also showed that the hof1ΔPEST protein accumulates instead of being degraded.   In order to study the effects of the Hof1 PEST domain phosphorylation mutants, time-lapse imaging of GFP tagged wild type or 10A hof1 was performed to monitor the dynamics of Hof1 at the bud neck during cytokinesis (Figure 1.5 and 1.6).

Figure 1.5. Ring Contraction in a Hof1 Wild Type Cell. Contraction of the Hof1 ring begins at 4 minutes and continues to 12 minutes. Bud rotation begins to occur at 30 minutes.

Figure 1.6. Ring Contraction in a Hof1 10A Cell. Contraction of the Hof1 ring begins at 6 minutes and continues to 14 minutes. Bud rotation begins to occur at 52 minutes.

Based on figures 1.5 and 1.6 it appears that the Hof1-GFP signal is brighter and more persistent in the mutant cell than in the wild type cells. In order to quantify the data Slidebook software was used to obtain the average fluorescence intensity measurements as described in methods. For the wild type (KSY 158) strain 7 cells were analyzed for the fluorescence intensity data at the bud neck and 5 cells were analyzed for the fluorescence intensity in the cytoplasm. For the 10A mutant (KSY 168) strain 3 cells were analyzed for the fluorescence intensity data at the bud neck and 2 cells were analyzed for the fluorescence intensity data in the cytoplasm. The fluorescence intensity data shows that the Hof1-GFP 10A is not being removed from the bud neck (Figure 1.7). This is shown because the fluorescence intensity values for both the bud neck (Figure 1.7) and cytoplasm (Figure 1.8) are staying around the same showing that there is not any movement or loss of hof1, suggesting that the phosphorylation of the Hof1 PEST domain is required to remove Hof1 from the bud neck after contraction. The fluorescence intensity data for the wild type shows that Hof1 is being removed from the bud neck after contraction; however it is not being degraded. Instead of being degraded the Hof1 protein is being relocalized to the cytoplasm of the cells. This can be seen because the Hof1 intensity is decreasing at the bud neck and increasing in the cytoplasm. By using time-lapse microscopy no effect was seen on double to single ring transition or on contraction of Hof1.
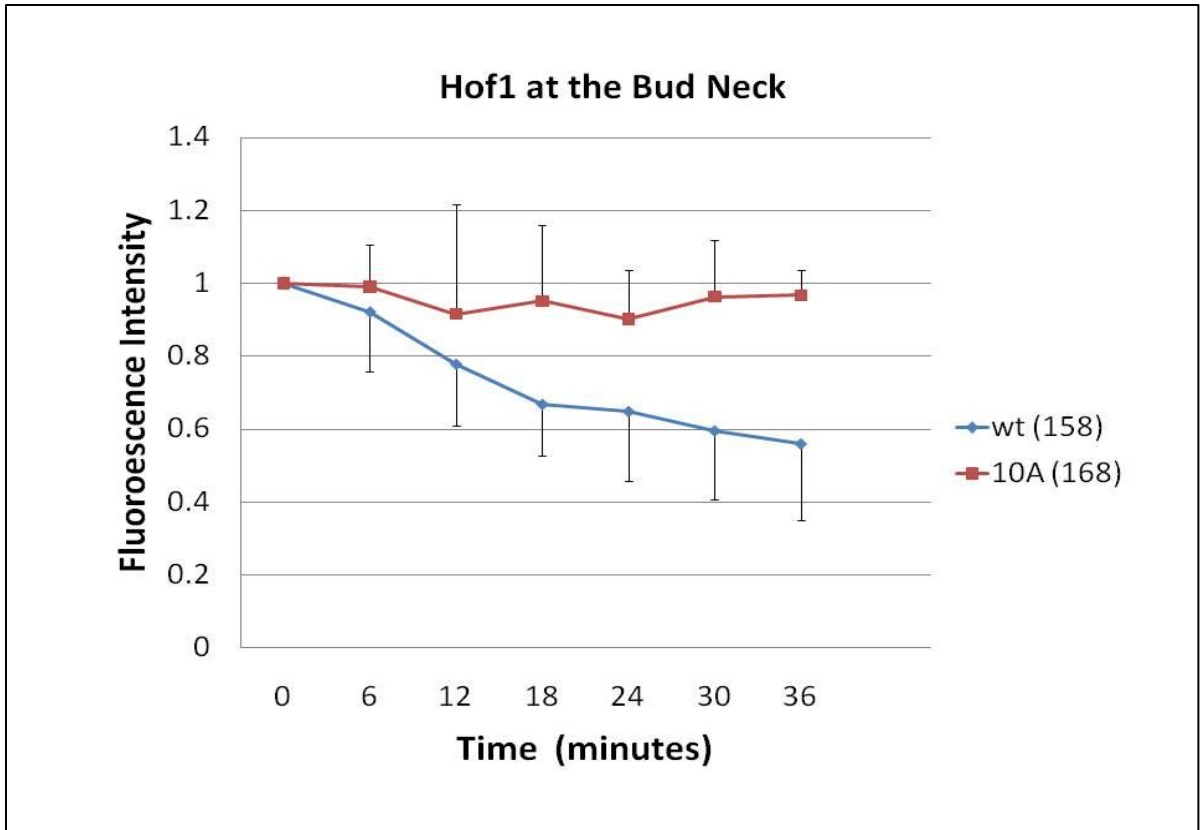
Figure 1.7. Normalized Average Fluorescence Intensity of Hof1-GFP at the Bud Neck.Time-lapse microscopy and Slidebook software were used to measure the mean intensity minus the background of Hof1-GFP wt and Hof1-GFP 10A (KSY 158 and KSY 168). Values were normalized to T=0, at the time point before Hof1 contraction.
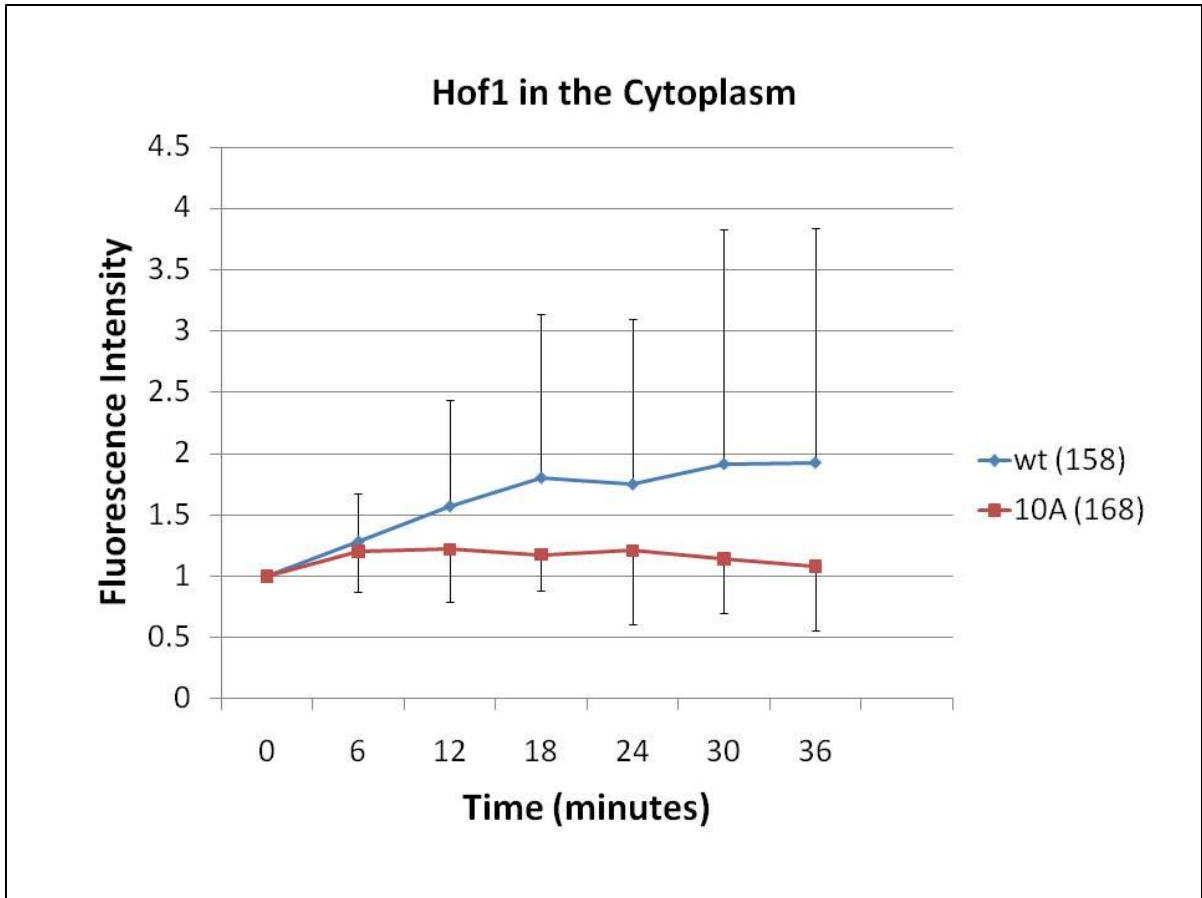
Figure 1.8. Normalized Average Fluorescence Intensity of Hof1 in the Cytoplasm. Time-lapse microscopy and Slidebook software were used to measure the mean intensity minus the background of Hof1-GFP wt and Hof1-GFP 10A (KSY 158 and KSY 168). Values were normalized to T=0, at the time point before Hof1 contraction.

3.3. PLASMID VS. CHROMOSOME TAGGED DOES NOT MAKE A DIFFERENCE IN HOF1 DEGRADATION

Previous research by Lippincott *et al* and Vallen *et al* had shown discrepancies between the localization of Hof1. Lippincott et al had shown that Hof1 was localized to the bud neck throughout the cell cycle (Lippincott and Li 1998), while Vallen *et al* had shown that Hof1 was not localized to the bud neck until G2/M (Vallen, Caviston et al. 2000). It was thought that these differences were because of the different constructs

that were used. Lippincott *et al* had used a Hof1 plasmid tagged with GFP at the C

terminus in their localization experiments (Lippincott and Li 1998), while Vallen *et al* had

fused their GFP tag to the 3' end of Hof1 chromosomal locus (Vallen, Caviston et al.

2000). Since it was obvious that the way the tag was attached could affect the

localization of Hof1, the question of whether or not the type of tag could affect the

degradation of Hof1 tested. In order to test if this was true two strains were obtained, a

Hof1-GFP wild type plasmid tagged (KSY 158) and a Hof1-GFP wild type chromosome

tagged (KSY 267). Both strains were then observed using time-lapse microscopy and

Slidebook software was used to obtain the average fluorescence intensity values at both

the bud neck (Figure 1.9) and in the cytoplasm (Figure 1.10).

Results from the graphs show the Hof1 wild type plasmid tagged (KSY 158) is

being removed from the bud neck but is accumulating in the cytoplasm instead of being

degraded. In the Hof1 wild type chromosome tagged (KSY 267) Hof1 is being removed

from the bud neck but is not increasing in the cytoplasm, showing that it is being

degraded.

3.4. GRR1 INTERACTION IS NOT DEPENDENT ON PEST DOMAIN PHOSPHORYLATION

The interaction between Hof1 and Grr1 is of interest because Grr1 is needed to

help degrade Hof1 after contraction. Previous research, including a genome-wide two

hybrid screen (Ito, Chiba et al. 2001) and a bimolecular fluorescence complementation

system (Blondel, Bach et al. 2005), have shown that Hof1 and Grr1 interact at the

mother bud neck. Previous research showed that the Hof1-Grr1 interaction is

dependent on the PEST domain (Blondel, Bach et al. 2005). Blondel *et al* created a

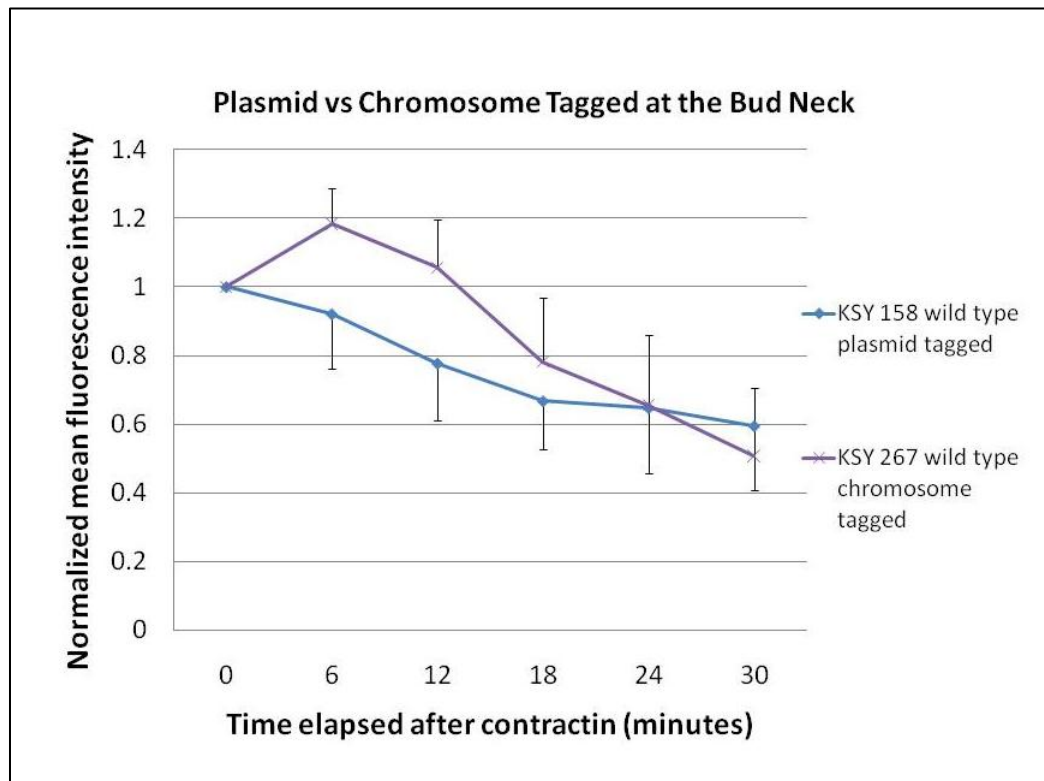Hof1ΔPEST strain, which was used to look at the Hof1-Grr1 interaction. In their

Figure 1.9. Normalized Average Hof1 Fluorescence Intensity Values in Wild Type Plasmid Tagged
(KSY 158) and Wild Type Chromosome Tagged (KSY 267) at the Bud Neck.Time-lapse microscopy
and SlideBook software were used to measure the mean intensity minus the background.
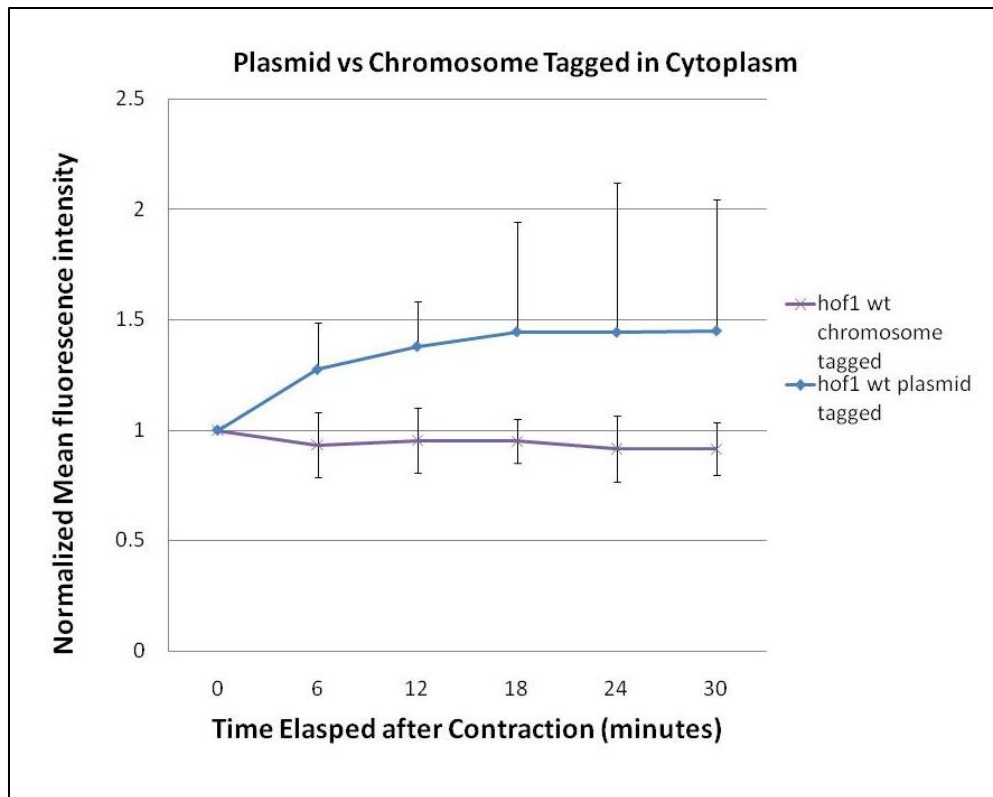Values were normalized to T=0, at the time point after Hof1 contraction.

Figure 1.10. Normalized Average Hof1 Fluorescence Intensity Values in Wild Type Plasmid Tagged (KSY 158) and Wild Type Chromosome Tagged (KSY 267) in the Cytoplasm.Time-lapse microscopy and Slidebook software were used to measure the mean intensity minus the background. Values were normalized to T=0, at the time point after Hof1 contraction.

experiments they saw a reduced interaction between Hof1ΔPEST and Grr1 in a two-

hybrid system as well as no expression of GFP in the bimolecular fluorescence

complementation system (Blondel, Bach et al. 2005). In a bimolecular fluorescence

complementation system fluorescence will be expressed if YFP-N and CFP-C are brought

together and fuse. In the experiment done by Blondel *et al* Grr1p-YFP-N and hof1ΔPEST-

CFP-C were coexpressed together, however the GFP was not expressed, suggesting that

they did not fuse (Blondel, Bach et al. 2005). In order to test to see if the Hof1-Grr1

interaction was dependent on phosphorylation of the PEST domain

co-immunoprecipitations and western blots on wild type (KSY 258) and 5A (KSY 259)

were performed as described in the design section, and results can be seen in Figure
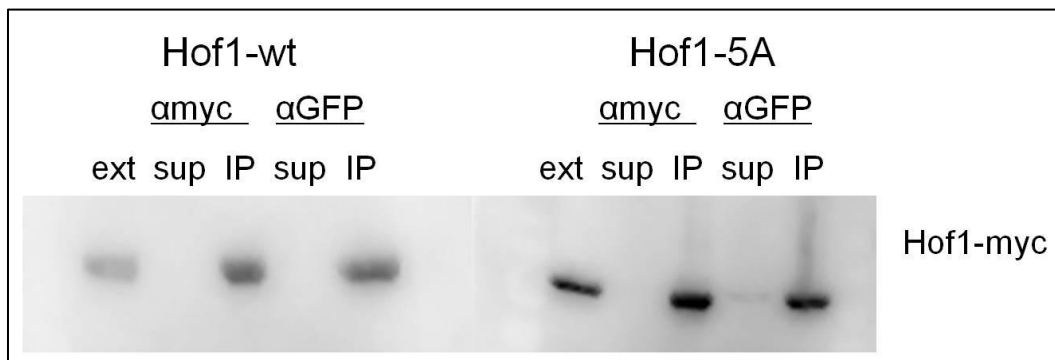
1.11 and 1.12.

Figure 1.11. Western Blot of Grr1-GFP-Immunoprecipitation(ext = extract, positive control; sup = supernatant, negative control; IP = immunoprecipitant). The proteins are precipitated using antibodies, the samples are then ran on a 7.5% SDS-page, transferred to nitrocellulose paper and incubated with anti-myc antibody. The results show that the hof1 (5A) mutant does not disrupt the Hof1-Grr1 interaction.

Figure 1.12. Western Blot of Hof1-myc-Immunoprecipitation (ext = extract, positive control; sup = supernatant, negative control; IP = immunoprecipitant). The proteins are precipitated using antibodies, the samples are then ran on a 7.5% SDS-page, transferred to nitrocellulose paper and incubated with anti-GFP antibody. The results show that the hof1 (5A) mutant does not disrupt the Hof1-Grr1 interaction.
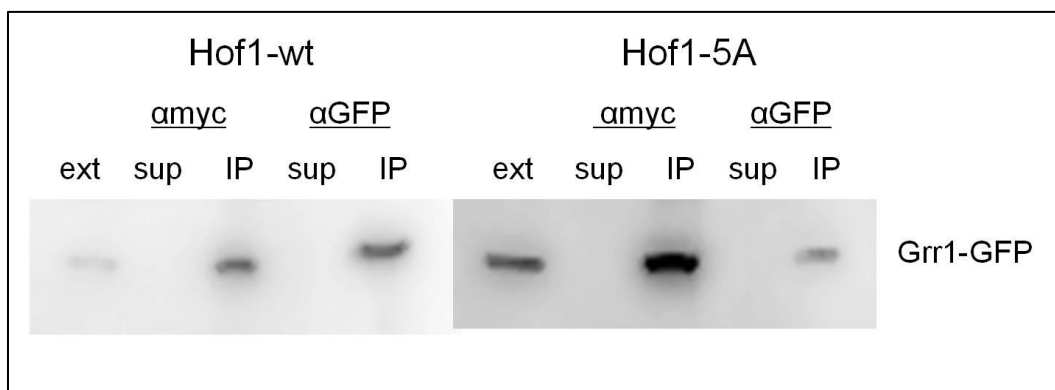
The results from the co-immunoprecipitations and western blots show that the Grr1 interaction is not dependent on phosphorylation of the PEST domain. This can be seen because in all of the IP lanes proteins can be seen. The wild type and 5A αmyc and αGFP IPs can be seen when blotting with either GFP or Myc antibodies, which show that Grr1 is still interacting with Hof1 even though the PEST domain has been mutated.

3.5. MYO1 CONTRACTION IS RELATED TO HOF1 PEST DOMAIN PHOSPHORYLATION

It is well known that Myo1 plays an important part in cytokinesis as part of the actomyosin ring. It is also known that the contraction of Myo1 is affected by Hof1. Lippincott and Li saw that the Myo1 rate of contraction in wild type cells was slower than that of ΔHof1 cells (Lippincott and Li 1998). Blondel *et al* has shown that in Hof1ΔPEST cells the Myo1 contraction was delayed compared to wild type cells (Blondel, Bach et al. 2005). Based off of the previous research and results an experiment was set up to look at the Myo1 rate of contraction in cells with a mutated PEST domain. Time lapse microscopy was performed on wild type, 5A mutated, and 10A mutated cells. Slidebook software was then used to determine the average size of the bud neck, average time of contraction and the average rate of contraction, results can be seen in Table 1.4.

This data suggests that phosphorylation of Hof1 PEST domain does play a role in the contraction of Myo1. The 10A mutant had the slowest average rate of contraction and the 5A had an intermediate average rate of contraction.

Table 1.4. Myo1 Contraction in wild type and mutant strains. An asterisk represents a P ≤ 0.05.

Strain	Average size of bud neck in µm +/- stdev	Average time of contraction in min +/- stdev	Average rate of contraction µm/min +/- stdev
Hof1 Wild type KSY 319	1.71	7.8	0.22
Hof1 5A KSY 268	1.32	8.0	0.16
Hof1 10A KSY 335	1.06*	7.67	0.14*

4. CONCLUSION

The goal of this study was to look at the effects of mutating the PEST domain of Hof1 to prevent phosphorylation. More specifically the effects on cytokinesis, degradation, contraction, and interaction were examined. Cell morphology analysis was done on wild type Hof1, Hof1Δ, Hof1 5A, and Hof1 10A strains and t-tests confirm that a cytokinesis defect did not result from mutating the Hof1 PEST domain. Time lapse microscopy and fluorescence intensity data analysis on wild type and 10A mutated strains show that phosphorylation of the Hof1 PEST domain is required to remove Hof1 from the bud neck after contraction. This can be seen because in the mutated strain the fluorescence intensity values did not change at either the bud neck or in the cytoplasm, however in the wild type strain the fluorescence intensity values decreased at the bud neck but increased in the cytoplasm. This also shows that wild type Hof1 is relocalized rather than degraded after ring contraction. When taken together this data shows that neither the removal nor degradation of Hof1 is needed for cytokinesis to occur.

The Hof1-Grr1 interaction was observed by using co-immunoprecipitations and western blots on wild type and mutated strains. In this experiment the Hof1-Grr1 interaction was not disrupted, showing the interaction is not dependent on the phosphorylation of the first 5 serines of PEST domain.

The contraction of Myo1-GFP was also observed by time lapse microscopy. In this experiment the wild type strain had the fastest rate of contraction and the mutated

10A had the slowest. This suggests that the PEST domain is involved in Myo1 contraction.

Overall the data shows that the PEST domain is involved in Hof1 removal from the bud neck and rate of Myo1 contraction, however even though these are being affected, a cytokinesis defect was not seen. A cytokinesis defect is most likely not seen because the mutations are making such a small change that they are not affecting the overall process.

5. FUTURE DIRECTIONS

To further support and prove the results some of the experiments will need to be repeated. For the fluorescence intensity data the 10A hof1 strain (KSY 168) has 3 cells that were analyzed at the bud neck and 2 cells in the cytoplasm, and ideally there should be 5 or more cells analyzed in both the bud neck and cytoplasm. Currently co-immunoprecitations and western blots are being performed on the 10A-Grr1 strain (KSY 330) and should be completed to see if the 10A mutant disrupts the Hof1-Grr1 interaction. After these experiments are finished up and the results are conclusive, future research opportunities can develop.

During the research, some important and new information about Hof1 has been shown; however there are still plenty of questions that need to be answered. One question is why is the wild type plasmid tag is not degraded and what is it doing? Another question is can Grr1 localize in Hof1Δ cells? A third question is how does the Hof1Δ or the PEST domain mutants affect other proteins and genes?

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VITA

Katherine Elizabeth Stockstill was born on December 31, 1986 in St. Louis, Missouri. In the spring of 2009 Katherine graduated with honors from Missouri University of Science and Technology with a B.S. in Biological Sciences. During her time as an undergraduate she was involved in research in the Cytokinesis Lab. She was also a member of the national biology honor society, Phi Sigma, as well a member of Kappa Delta Sorority. In the spring of 2011, Katherine earned her Master's degree in Applied and Environmental Biology from Missouri University of Science and Technology.

Throughout her time doing research, Katherine has had the opportunity to attend and present her research at several conferences and meetings including: UMR Undergraduate Research Conference, Missouri S&T Undergraduate Research Conference, Missouri S&T Graduate Research Showcase, Central States Microscopy and Microanalysis Society Meeting, Midwest Yeast Meeting, and the American Society of Cell Biology Conference. Katherine has also had the opportunity to be published.